



Characterization of the virome in broad bean: First detection of *Potyvirus phaseoluteum* and *Orthotospovirus impatiensnecromaculae* in Montecillo, Texcoco, Mexico

Erick Ortega-Piña, Daniel Leobardo Ochoa-Martínez*, Reyna Isabel Rojas-Martínez; Posgrado en Fitosanidad - Fitopatología. Colegio de Postgraduados, Carretera Federal México-Texcoco, Km. 36.5. Montecillo, 56264 Texcoco, Estado de México, México; Alfredo Díaz-Lara, Departamento de Bioingeniería. Tecnológico de Monterrey Campus Querétaro. Epigmenio González 500, San Pablo, Santiago de Querétaro, México.

***Corresponding Author:**

Daniel Leobardo
Ochoa-Martínez
ldaniel@colpos.mx

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ABSTRACT

Background / Objective. In broad bean (*Vicia faba*) crops established in Montecillo, Texcoco, State of Mexico, 100% of plants were observed with viral symptoms consisting of mosaic, mottling, leaf curling, wilting and a notable decrease in plant development. These symptoms led to a significant reduction in seed yield and quality. In this context, the present study was carried out with the objective of identifying the viral species associated with these symptoms.

Materials and Methods. The experimental strategy consisted of extraction of total RNA from leaves showing the aforementioned symptoms, followed by next-generation sequencing.

Results. In the results, the complete genomes of *Orthotospovirus impatiensnecromaculae* (before impatiens necrotic spot virus) and two isolates of the *Potyvirus phaseoluteum* (before bean yellow mosaic virus) were obtained. The phylogenetic analysis revealed that the isolate of *O. impatiensnecromaculae* (INSV CPMEX) shows significant divergences from those previously reported in other plant species. On the other hand, the two *P. phaseoluteum* isolates (BYMV CP1MEX and BYMV CP2MEX) proved to be different from each other, being related to isolates reported in Sudan and Iran.

Conclusion. This result suggests considerable genetic diversity among the viruses associated with viral symptoms in faba bean crops in the region, which underlines the importance of accurate identification for the management and control of these viral infections.

Keywords: Next -Generation Sequencing, *Potyvirus*, *Orthotospovirus*, RNA, mosaic, mottle, viral divergence.

INTRODUCTION

On a global scale, the broad bean crop currently covers 2.5 million hectares with a production of 5.4 million tons and an average seed yield of 2.1 tons/ha (FAOSTAT, 2023). This crop is not only valuable for its nutritional and medicinal properties that offer significant benefits for human health (Prabhu and Rajeswari, 2018), but also for its economic contribution. However, plant viruses also represent a considerable threat to their production (Mansourpour *et al.*, 2022); (Makkouk *et al.*, 1994).

The adoption of the PCR (Polymerase Chain Reaction) and other viral detection techniques revealed the complexity of the viral etiologies in plants (Binnicker, 2015). The introduction of Next-Generation Sequencing (NGS) has revolutionized genomic investigation using massive biological data, allowing for the analysis of vast amounts of sequences in an efficient way (Rubio *et al.*, 2020). This technology has facilitated the advanced diagnosis and the identification of viromes, i.e., the set of viruses and viroids found in infected plants or their vectors, opening new options for their management (Barba *et al.*, 2014).

In this context, the present study focused on broad bean plantations in Montecillo, Texcoco, State of Mexico, where a total prevalence of virosis was observed, characterized by symptoms such as mosaic, mottle, leaf curling, wilting and growth reduction. These symptoms cause a significant reduction in seed yield and quality. The aim of this investigation was to determine the virome associated to these symptoms, with the intention of contributing to the development of more effective management strategies for this crop.

A random sampling was conducted on 95-day-old broad bean plants showing symptoms of mottle, leaf curling and wilting in a plot located in Montecillo, Texcoco, State of Mexico. Leaves from the upper third of 10 plants were collected and kept refrigerated at 4 °C until the RNA extraction process. For the extraction of total RNA, 0.1 g of leaf tissue from each plant was macerated with liquid nitrogen until a fine powder was obtained, using the SV Total RNA Isolation System® PROMEGA kit, following the instructions by the manufacturer. The quality and concentration of the total RNA extracted was quantified by spectrophotometry (Nanodrop 2000®, Thermo Scientific™). To verify its integrity, electrophoresis was carried out at 90 V for 30 minutes in a 1% agarose gel with ethidium bromide visualized in a UV light photodocumenter.

In order to validate the results obtained through massive sequencing, specific primers were designed, focusing on the genomic segments that encode the viral

replicase and the coat protein (Table 1. List of the primers used). The selection of these segments as targets for the design of the primers is due to their functional importance and their conservation among different virus isolates, making them ideal regions for the precise detection of the plant pathogen. Once the primers were designed, the reverse transcription polymerase chain reaction (RT-PCR) was performed for each one of the individual samples that were originally combined to form the compound sample analyzed by massive sequencing, as well as the rest of the samples that were kept to analyze the presence or absence of the species found.

Table 1. Primers designed to verify the presence of *Potyvirus phaseoliteum* (Pp) and *Orthotospovirus impatiensnecromaculæ* (Oi) in broad bean foliar tissue with symptoms of mosaic, mottling, leaf curling, wilting and growth reduction.

Virus	Primers	Amplified region	Amplicon
Oi	S: 5'CAGCCGCTTCTTCACTCTGT3' As: 5'TGTGAGTTTCTCGGAAGGCT3'	Viral replicase	438 pb
Pp	S: 5'AATGCGAAGCCAACATTCCG3' As: 5'CACGGTTGACATCTCCTGCT3'	Coat protein	319 pb

The mixture for the cDNA synthesis of the viruses found consisted of 5.3 µL of nuclease-free water, 0.5 µL of each primer and 1 µL of total RNA for a final volume of 7.3 µL, which were incubated for 5 minutes at 70 °C, and after this step was completed, the tubes were placed in ice. Subsequently, a mixture was prepared with 2 µL of RT-5X buffer, 0.5 µL of dNTP Mix and 0.2 µL of M-MLV Reverse Transcriptase; 2.7 µL of this mixture were added to each reaction tube incubated in the first step to obtain a total of 10 µL. The tubes remained for 60 minutes at 42 °C for 10 minutes at 72 °C in the thermocycler. For the PCR reaction, the mixture consisted of 2 µL of 5X Green GoTaq® buffer, 0.6 µL of MgCl₂ (25 mM), 0.2 µL of dNTP Mix (10 mM), 0.6 µL of each primer (10 pmol/µL), 4.9 µL of nuclease-free water, 0.1 µL of GoTaq® Flexi Polymerase (5u/µL) and 1 µL of cDNA for a total volume of 10 µL per reaction. The conditions for amplification consisted of an initial 5-minute denaturalization reaction at 95 °C, 35 cycles for 30 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C, followed by a final 10-minute extension at 72 °C. The PCR product was separated by electrophoresis at 80 V for 55 minutes in 1% agarose gel stained with ethidium bromide and was visualized in a UV light photodocumenter. The amplicons obtained were sent to Macrogen Inc. (Korea) for sequencing and later comparison with those deposited in the GenBank with BLASTn.

For each one of the viruses found (*P. phaseoliteum* and *O. impatiensnecromaculæ*), two analyses were performed: one for the segment that codifies the viral replicase and the other, for the one that codifies the nucleocapsid. The complete virus genomes were aligned using ClustalW in MEGA X. This alignment included complete genome sequences corresponding to viruses from the same species, from a different species but within the same genus, and from a species of a different genus. The inclusion of these sequences, all available in the NCBI database, provides a comparative framework that helps interpret the evolutionary relationships between the viruses studied. The construction of the phylogenetic tree was based on the application of sophisticated evolutionary models, specifically the General-Time-Reversible (GTR) and the Jones Taylor Thornton (JTT) models. These were selected for their ability to accurately estimate the base substitution rates over evolutionary time, offering a detailed view of phylogenetic relationships. Both were constructed using the maximum likelihood method with 500 bootstrap replicates in MEGA X. This statistical approach allows for the evaluation of the robustness of each node in the tree, providing an indicator of the confidence in the observed phylogenetic groupings.

The quality evaluations on the RNA samples revealed that they were within the optimal purity ranges, with 260/280 absorbance ratios between 1.8 and 2.2, and 260/230 also within the ideal interval, which indicates a minimal presence of organic and inorganic contaminants. Additionally, the concentration of the extracted RNA was greater than 300 ng/µL, surpassing the threshold required to ensure a sufficient number of subsequent analyses. Likewise, in the electrophoresis gel for integrity, clear stripes without smearing due to chain fragmentation were observed, indicating optimal integrity.

Two complete *Potyvirus phaseoliteum* genomes were obtained, one of 9,577 nt and the other of 9,575 nt corresponding to different isolates, denoted as CP1 MEX and CP2 MEX, respectively; likewise, the three complete segments of the *Orthospovirus impatiensnecromaculæ* genome were obtained, whose lengths were as follows: segment L (viral replicase) 8,763 nt, segment M (movement protein) 3,629 nt and segment S (nucleocapsid protein and NSs protein) 1,825 nt. In all three cases, the entire codifying regions were found and deposited in the GenBank with accession numbers PP098739 (BYMV CP1MEX), PP098740 (BYMV CP2MEX), PP098741 (INSV CPMEX L), (PP098742) INSV CPMEX M and PP098743 (INSV CPMEX S).

The amplicons corresponding to the expected size for each virus in the field samples were obtained (Figure 1) with the primers designed for the two *P. phaseoliteum* and *O. impatiensnecromaculæ* isolates found in this investigation (Table 1).

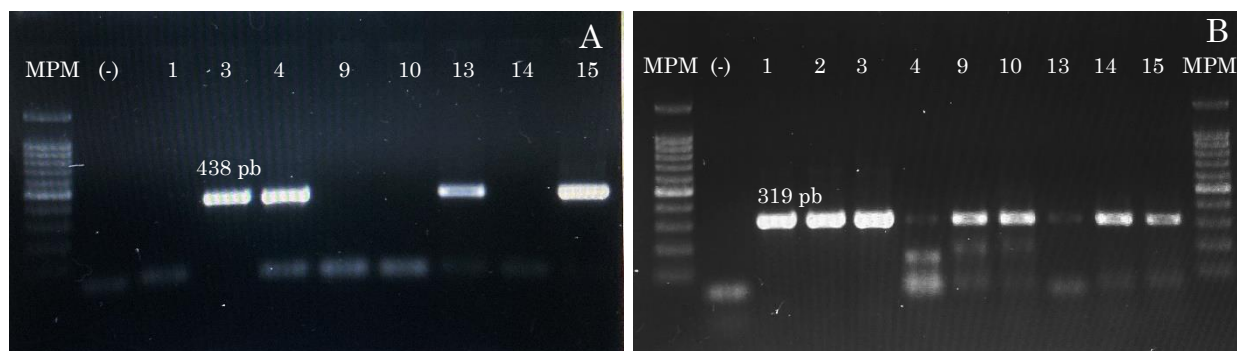


Figure 1. Detection of *Orthotospovirus impatiensnecromaculæ* and *Potyvirus phaseoluteum* in broad bean plants using RT-PCR. A) Product of RT-PCR of a 438 bp fragment of the L segment that codifies the viral replicase of *O. impatiensnecromaculæ*: MPM= Molecular weight marker 100 bp PROMEGA, negative control (-). Lanes 1, 3, 4, 9, 10, 13, 14 and 15 broad bean plant samples with symptoms of mosaic, mottling, leaf curling and wilting. B) Product of RT-PCR of a 319 bp fragment of the ORF of the *P. phaseoluteum* that codifies for the nucleocapsid MPM= Molecular weight marker 100 bp PROMEGA, negative control (-). Lanes 1, 2, 3, 4, 9, 10, 13, 14 and 15 broad bean plant samples with symptoms of mosaic, mottling, leaf curling and wilting. The sequences of the amplicons obtained had a 99% coverage and 99% identity with *P. phaseoluteum* and *O. impatiensnecromaculæ* isolates deposited in the GenBank, respectively.

The BYMV isolates found in broad beans in this investigation are grouped with those from Sudan and Iran, respectively, but not amongst themselves (Figure 2).

The relatedness of the *Potyvirus phaseoluteum* isolates with those from the Asian countries suggests that the virus has been spread to other continents, most likely due to the mobilization of seeds, since this is the main means of propagation of the broad beans, and constitutes a common way of transmission of the species of the *Potyvirus* genus (Díaz *et al.*, 2010). Thus, once the source of inoculum is present in the plantation, its dissemination can take place via different species of aphids (Rodríguez-Pardina *et al.*, 2019), which would explain the high incidence of plants with symptoms found in the field. There is no information on the presence of *P. phaseoluteum* in Mexico, making this its first report. On the other hand, the

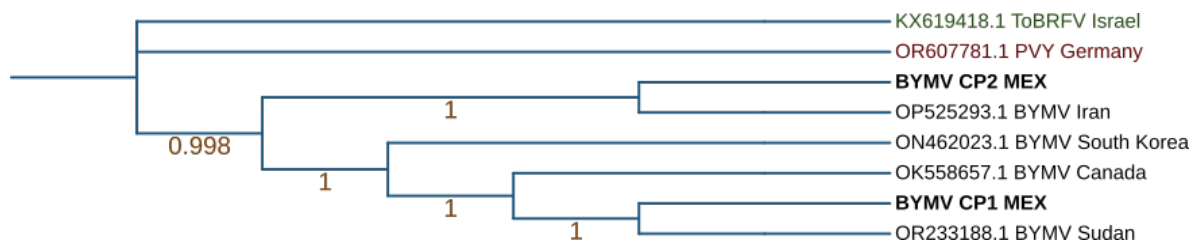


Figure 2. Phylogenetic tree of the *Potyvirus phaseoluteum* CP1MEX and BYMV CP2MEX isolates using the General-Time-Reversible model and the maximum likelihood method with 500 bootstrap replicates.

sequences of INSV CPMEX segments L and S obtained in this study (Figures 3 and 4) were not grouped with those reported in other countries, suggesting that this is a highly divergent isolate, which may be attributed to its adaptation to the geographic space in which it is found, derived from the changes it has experienced with time in the region under study.

O. impatiensnecromaculae mainly infects ornamental species, and in Mexico it has also been reported in tomato and chili pepper (González-Pacheco and Silva-Rosales, 2013). An earlier study different plant species were inoculated with *O. impatiensnecromaculae* and the broad bean was found to be a host of this virus (Abe El-Wahab and Elnagar, 2008). By contrast, this investigation reports the natural infection of this virus in broad beans.

Worldwide, *Fabavirus alphaviciae* (before broad bean wilt virus) (Balsak *et al.*, 2023; Xia *et al.*, 2020), *Bromovirus BBMV* (before broad bean mottle virus) (Makkouk *et al.*, 1988; Romero *et al.*, 1993), *Luteovirus phaseoli* (before bean leaf roll virus) (Makkouk *et al.*, 2012), *Potyvirus phaseoluteum* (before bean

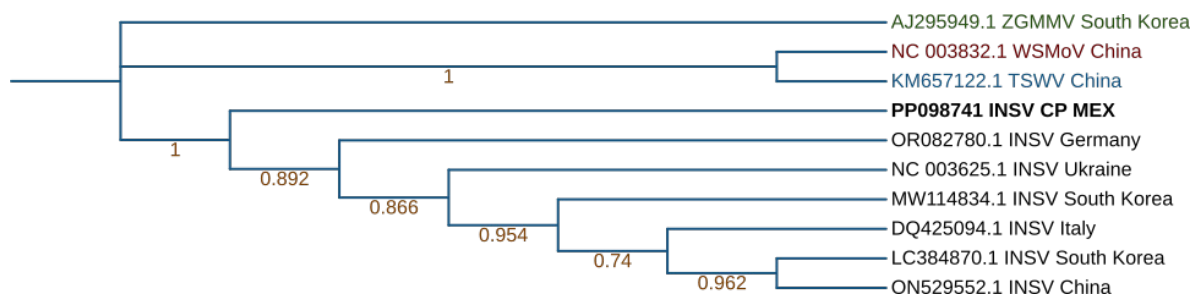


Figure 3. Phylogenetic tree of the L segment corresponding to the viral replicase of the *Orthotospovirus impatiensnecromaculae* INSV CP MEX isolate using the Jones Taylor Thornton Model and the maximum likelihood method with 500 bootstrap replicates.

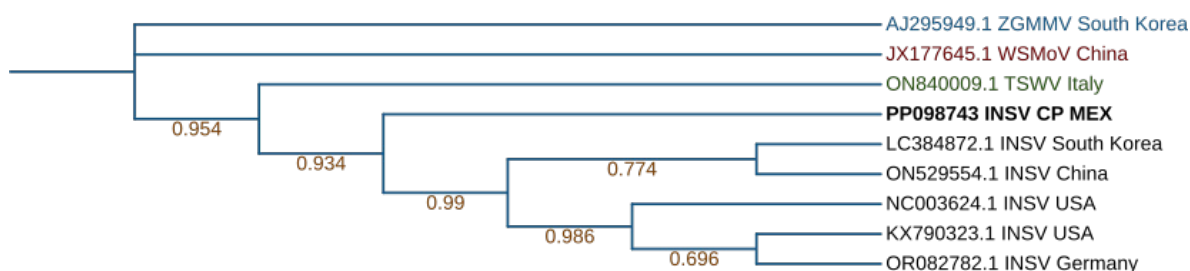


Figure 4. Phylogenetic tree of the S segment corresponding to the nucleocapsid of the INSV CP MEX isolation of the *Orthotospovirus impatiensnecromaculae* using the Jones Taylor Thornton Model and the maximum likelihood method with 500 bootstrap repetitions.

yellow mosaic virus) (Chatzivassiliou, 2021; El-Helaly *et al.*, 2016) and *Nanovirus necroflaviviciae* (before faba bean necrotic yellows virus) (Mansourpour *et al.*, 2022; Katul *et al.*, 1993) have been reported as main species that naturally infect broad beans, out of which only *P. phaseoliteum* and *O. impatiensnecromaculae* were found in this study. Based on these results, it would be necessary to study the virome of the broad bean in other production areas to know the virus diversity associated with this crop in the country.

The study of the virome found in broad bean crops in Montecillo, Texcoco, State of Mexico, has revealed a specific composition of viral plant pathogens, which consist of two different *Potyvirus phaseoliteum* isolates (designated as BYMV CP1MEX and BYMV CP2MEX), associated to mosaic and mottle symptoms, and an *Orthotospovirus impatiensnecromaculae* isolate (INSV CP MEX) associated to leaf curling and wilting in a mixed infection with *P. phaseoliteum*. This is the first report of *P. phaseoliteum* and of the natural infection by *O. impatiensnecromaculae* in broad bean plantations in Mexico. These findings provide crucial evidence, highlighting the importance of monitoring and managing these viral infections to protect the agricultural production of broad beans in the region.

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